An integrated clinico-transcriptomic approach identifies a central role of the heme degradation pathway for septic complications after trauma

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Abstract: OBJECTIVE: The present study was aimed to identify mechanisms linked to complicated courses and adverse events after severe trauma by a systems biology approach. SUMMARY BACKGROUND DATA: In severe trauma, overwhelming systemic inflammation can result in additional damage and the development of complications, including sepsis. METHODS: In a prospective, longitudinal single-center study, RNA samples from circulating leukocytes from patients with multiple injury (injury severity score 17 points; n = 81) were analyzed for dynamic changes in gene expression over a period of 21 days by whole-genome screening (discovery set; n = 10 patients; 90 samples) and quantitative RT-PCR (validation set; n = 71 patients, 517 samples). Multivariate correlational analysis of transcripts and clinical parameters was used to identify mechanisms related to sepsis. RESULTS: Transcriptome profiling of the discovery set revealed the strongest changes between patients with either systemic inflammation or sepsis in gene expression of the heme degradation pathway. Using quantitative RT-PCR analyses (validation set), the key components haptoglobin (HP), cluster of differentiation (CD) 163, heme oxygenase-1 (HMOX1), and biliverdin reductase A (BLVRA) showed robust changes following trauma. Upregulation of HP was associated with the severity of systemic inflammation and the development of sepsis. Patients who received allogeneic blood transfusions had a higher incidence of nosocomial infections and sepsis, and the amount of blood transfusion as source of free heme correlated with the expression pattern of HP. CONCLUSIONS: These findings indicate that the heme degradation pathway is associated with increased susceptibility to septic complications after trauma, which is indicated by HP expression in particular.

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An Integrated Clinico-transcriptomic Approach Identifies a Central Role of the Heme Degradation Pathway for Septic Complications After Trauma

Daniel Rittirsch, MD, Veit Schoenborn, MD,* Sandro Lindig, PhD,† Elisabeth Wanner, MD,* Kai Sprengel, MD,* Sebastian Günkel, MD,* Markus Blaess, PhD,†‡ Barbara Schaarschmidt, MSc,†‡ Patricia Sailer,* Sonja Märsmann,* Hans-Peter Simmen, MD,* Paolo Cinelli, PhD,* Michael Bauer, MD,†‡ Ralf A. Claus, PhD,†‡ and Guido A. Wanner, MD**

Objective: The present study was aimed to identify mechanisms linked to complicated courses and adverse events after severe trauma by a systems biology approach.

Summary Background Data: In severe trauma, overwhelming systemic inflammation can result in additional damage and the development of complications, including sepsis.

Methods: In a prospective, longitudinal single-center study, RNA samples from circulating leukocytes from patients with multiple injury (injury severity score $\geq$17 points; n = 81) were analyzed for dynamic changes in gene expression over a period of 21 days by whole-genome screening (discovery set; n = 10 patients; 90 samples) and quantitative RT-PCR (validation set; n = 71 patients, 517 samples). Multivariate correlational analysis of transcripts and clinical parameters was used to identify mechanisms related to sepsis.

Results: Transcriptome profiling of the discovery set revealed the strongest changes between patients with either systemic inflammation or sepsis in gene expression of the heme degradation pathway. Using quantitative RT-PCR analyses (validation set), the key components haptoglobin (HP), cluster of differentiation (CD) 163, heme oxygenase-1 (HMOX1), and biliverdin reductase A (BLVRA) showed robust changes following trauma. Upregulation of HP was associated with the severity of systemic inflammation and the development of sepsis. Patients who received allogeneic blood transfusions had a higher incidence of nosocomial infections and sepsis, and the amount of blood transfusion as source of free heme correlated with the expression pattern of HP.

Conclusions: These findings indicate that the heme degradation pathway is associated with increased susceptibility to septic complications after trauma, which is indicated by HP expression in particular.

From the *Division of Trauma Surgery, Department of Surgery, University Hospital Zurich, University of Zurich, Zurich, Switzerland; †Department of Anaesthesiology and Intensive Care Therapy, Jena University Hospital, Jena, Germany; and ‡Center for Sepsis Control and Care, Jena University Hospital, Jena, Germany.

All authors contributed equally.

All authors jointly supervised this study.

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Reprints: Guido A. Wanner, Division of Trauma Surgery, Department of Surgery, University Hospital Zurich, Raemistrasse 100, CH-8091 Zurich, Switzerland. E-mail: guido.wanner@usz.ch.

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Multiple trauma still represents one of the leading causes of death in western countries, and treatment of severely injured patients imposes a considerable burden for healthcare systems.[1–3] Severe trauma triggers systemic inflammation, the clinical presentation of which is indistinguishable from sepsis syndrome, but not necessarily associated with infection.[4,5] Already decades ago, it has been demonstrated that multiorgan failure (MOF) frequently occurred in trauma patients without infection.[6] While the overall mortality rate from multiple trauma has declined significantly, the incidence of secondary sepsis after trauma remained unchanged during the past decade and represents a dreaded complication with prolonged necessity of intensive care and an unfavorable outcome.[1] The incidence of MOF induced by traumatic shock has decreased substantially, whereas a decrease in MOF incidence due to sepsis is not observed.[1] The decrease in inhospital mortality from severe trauma was inter alia achieved because the underlying pathophysiology is reflected by current treatment concepts, including damage control surgery and evidence-based protocols for fluid resuscitation, blood transfusion, and the management of traumatic coagulopathy.[7–9] With the definition of sepsis criteria in the 1990s, it has been conceptualized that the immune response after trauma follows a biphasic course with an early hyperinflammation (systemic inflammatory response syndrome; SIRS) that is terminated by immunosuppression during the later course (compensatory anti-inflammatory response syndrome).[10,11] In this concept, MOF was thought to be either induced by an overwhelming initial insult (''one-hit model'') or SIRS was amplified by a second insult (eg, surgical intervention; ''two-hit model'').[5,10,12] Recently published findings of the Glue Grant Consortium on leukocyte genomic expression patterns showed the simultaneous induction of a plethora of pro- and anti-inflammatory genes after trauma, whereas adaptive immunity-associated genes were found to be suppressed. Based on these data, a novel model (''genomic storm'') has been derived, which challenges the second hit theory of the traditional SIRS—compensatory anti-inflammatory response syndrome paradigm, as the response of patients with different courses after trauma was found to be more common than different.[5,13] Although recent research has improved our understanding of the network of systemic inflammation, the mechanisms that lead to adverse events, including nosocomial infections, MOF, and sepsis are inadequately understood, and specific immune-modulatory therapies are still far from reach for the treatment of trauma patients.[12,14]

In the present study, we aimed to investigate mechanisms of systemic inflammation after severe trauma by analysis of leukocyte transcriptomic expression patterns in order to identify pathways that are associated with infectious complications and sepsis, in particular.

METHODS

Study Design

Blood was sampled from 104 patients with multi-system trauma admitted to the Division of Trauma Surgery (level I trauma center) at the University Hospital Zurich from 12/2009 to 03/2012. Criteria for study enrollment included patient age ≥18 years, an Injury Severity Score (ISS) ≥17 points, and time from injury to admission <6 hours. All patients were recruited into the study under informed consent guidelines approved by the Local Ethical Committee (StV 26–2007) and international ethical guidelines (ClinicalTrials.gov-Identifier: NCT02508272). Study subjects were treated under the guidance of standard operating procedures developed and implemented at the University Hospital Zurich (based on guidelines of the German Society of Trauma; DGU).[15] Whole blood from trauma patients was collected within the first 6 hours after trauma (day 0) and on days 1, 2, 3, 5, 7, 10, 14, and 21. Clinical outcomes and complications within 28 days after injury were recorded. Patient enrollment and study design are illustrated in Supplemental Fig. 1, http://links.lww.com/SLA/A929. For discovery, 10 representative patients (discovery set; n = 90 samples) were selected based on their clinical presentation with respect to the development of secondary sepsis (n = 5 patients; n = 45 samples) or systemic inflammation without infection (n = 5 patients; n = 45 samples) for whole-genome screening. After statistical gene selection, results were validated in the remaining patients of the total cohort (validation set; n = 71 patients; n = 517 samples) by quantitative RT-PCR.

Clinical Data

Clinical data were prospectively collected in parallel with the corresponding blood samples. The occurrence and severity of systemic inflammation, sepsis, MOF, and nosocomial infections were retrospectively analyzed using the corresponding clinical parameters from patients' records. MOF was defined according to the Sequential Organ Failure Assessment (SOFA) score.[16] Systemic inflammation and sepsis was defined according to criteria of the current guidelines.[17–19] For assessment of the severity of trauma-induced systemic inflammation and to define secondary sepsis in trauma patients a scoring system has been applied (SI score; for details see Supplemental Information, Supplemental Table 1 and Supplemental Fig. 2, http://links.lww.com/SLA/A929).[20–24]

RNA Isolation

PaxGene (PreAnalytix, Hombrechtikon, Switzerland) tubes were used for sampling and preservation of whole blood, and total cellular RNA from circulating leukocytes was isolated (PaxGene Blood RNA Kit; PreAnalytix) in a Qiacube apparatus according to the manufacturer's instructions. RNA integrity was proven using Experion (Biorad, Munich, Germany) microcapillary electrophoresis. A detailed description of the totRNA quality control and sample selection criteria is provided in Supplement, http://links.lww.com/SLA/A929. For cDNA-synthesis, 1 μg total RNA per sample was transcribed (RevertAid First Strand cDNA Synthesis, ThermoFisher Scientific; PTC-200 Thermal Cycler Dual, BioRad) according to manufacturer's protocol.

Microarray Analysis

A total of 200 ng RNA of each sample was reversely transcribed and amplified using the TargetAmp-Nano Labeling Kit for Illumina Expression BeadChips (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) according to the manufacturer's instructions on a Biorad MJ Thermal Cycler (Biorad). All cRNA samples were purified the NucleoSpin RNA Clean-up system (Macherey-Nagel, GmbH & Co. KG, Düren, Germany) and quantified using a NanoDrop spectrophotometer ND-2000 (Thermo Fisher Scientific, Schwerte, Germany/PEQLAB Biotechnologie, Carlsbad, CA) before proceeding to sample hybridization. Samples were prepared and hybridized on Illumina Human-HT-12-V4 Expression BeadChips (Illumina, San Diego, CA, USA; for details see Supplemental Information, http://links.lww.com/SLA/A929). For posthybridization data read-out and data preprocessing, including spot detection, gene mapping, and averaging of replicates, iScan Control Software was used. Control probe quality check was performed using Illumina Genome Studio-Software.

Statistical analysis (for details see Supplement, http://links.lww.com/SLA/A929) was performed using R software (http://www.r-project.org/) and packages from Bioconductor.[25] Differentially expressed genes were filtered by exhibiting above median-averaged 1.5-fold change, estimate of significance $P \leq 0.05$ and a false discovery rate <0.05. Candidate genes selected for further analysis and validation by quantitative real-time PCR were identified by gene-set enrichment analysis (list is provided in Supplemental Table 5, http://links.lww.com/SLA/A929).

Quantitative RT-PCR

RT-qPCR was performed in a two-step protocol using Rotor-Gene system and Rotor-Gene SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to manufacturer's information with 250 nM Primer mix and 25 ng cDNA. Initial denaturation at 95°C for 5 minutes, followed by 50 cycles of denaturation at 95°C for 5 seconds and annealing/extension at a given temperature (see table) for 15 seconds, finally followed by a melting curve. Ct values and efficiency were documented for each sample, data were normalized to unchanged transcripts, whereby housekeeper adjustment (ΔCt) was performed via expressed ACTB [ΔCt = Ct(ACTB) - Ct(Candidate)]. The primer sequences are listed in Supplemental Table 2, http://links.lww.com/SLA/A929. No Ct values of genes with significant increases/decreases as well as reference genes included in this study were detected above 26 cycles. Comparisons of PCR values for the various groups are displayed in Box-and-Whisker plots. Significance was attained at $P < 0.05$ using the Mann-Whitney U (Wilcoxon rank sum) test. Similarity was assessed using parametric (r) and nonparametric (ρ) measures. Influences of clinical variables and their interactions on gene expression were investigated using multivariate ANOVA.

RESULTS

Transcriptome Profiling for Hypothesis Generation

Characteristics of the patient cohort are displayed in Table 1 (for further details see Supplemental Information and Supplemental Table 3, http://links.lww.com/SLA/A929).

In a discovery set, RNA samples from 10 representative trauma patients (all time points) diagnosed with either secondary sepsis or systemic inflammation without infection, respectively, were analyzed by whole-genome screening of differential gene expression patterns. Using unsupervised clustering, patients with systemic inflammation only and sepsis patients showed a distinct expression pattern and the discrimination of clinical presentation (Fig. 1A). Explorative gene-set analysis revealed robust upregulation of genes related to ''hemoglobin metabolism/oxygen transport'' and ''pathogenic *Escherichia coli* infection,'' whereas the strongest downregulation was found for ''ribosome-associated genes'' (Table 2).

TABLE 1. Patient Characteristics

Parameter	Total Cohort (n = 104 patients)	Discovery Set (n = 10 patients)	*P*
Demographics			
	mean ± SEM; median (min.-max.)	mean ± SEM; median (min.-max.)	
Age (yr)	43.5 ± 1.77; 42 (18–92)	41 ± 4.9; 42 (20–62)	0.85; ns
Sex (male/female)	77/27	8/2	
GCS	11.9 ± 0.41; 14 (3–15)	12.8 ± 1.2; 14.5 (5–15)	0.58; ns
Traumatic brain injury	38/104 (36.5%)	2/10 (20%)	
AIS max.	4.1 ± 0.1; 4 (2–6)	4.6 ± 0.2; 5 (3–5)	
AIS Head/neck/cervical spine	2.2 ± 0.2; 2 (0–6)	1.5 ± 0.5; 1 (0–5)	
AIS Face	0.7 ± 0.1; 0 (0–3)	0.5 ± 0.2; 0 (0–2)	
AIS Thorax/thoracic spine	2.9 ± 0.2; 3 (0–5)	3.3 ± 0.5; 3.5 (0–5)	
AIS Abdomen/lumbar spine	1.9 ± 0.2; 2 (0–5)	3.2 ± 0.2; 3.5 (0–5)	
AIS Upper/lower Extremitiy	2.4 ± 0.1; 3 (0–5)	2.5 ± 0.4; 3 (0–5)	
AIS Integument	0.4 ± 0.1; 0 (0–3)	0.7 ± 0.3; 0 (0 -2)	
ISS	32.8 ± 1.3; 31 (17–75)	37.5 ± 2.3; 36 (27–50)	0.07; ns
SOFA score initial	4.8 ± 0.3; 5 (0–12)	5.5 ± 0.9; 6.5 (0–9)	0.45; ns
SOFA score max.	7.7 ± 0.4; 8 (0–18)	10.9 ± 1.4; 12 (1–16)	<0.05
Outcomes			
RISC (% survival)	83.3 ± 2.5	88.6 ± 3.5; 94.2 (71.1–97.6)	0.79; ns
Survival	88% (13 nonsurvivors)	100%	
Hospital length of stay (d)	26.6 ± 1.8; 21.5 (2–119)	40.4 ± 6.0; 42.5 (17–82)	<0.01
Intensive care unit length of stay (d)	13.9 ± 1.4; 10 (2–86)	27.4 ± 7.4; 19.5 (6–86)	<0.05
Allogeneic blood transfusion			
TASH score (points)	8.5 ± 0.6; 8 (0–23)	11.3 ± 2.5; 10 (2–22)	0.22; ns
TASH (%)	13.9 ± 2.0	22.1 ± 10.6; 8 (5–77)	0.14; ns
Initial (d0) pRBC transfusion (units)	4.0 ± 0.8; 1 (0–54)	11.3 ± 5.3; 4 (0–54)	<0.05
Total pRBC transfusion (units)	9.6 ± 1.3; 5 (0–70)	21.2 ± 8.0; 9 (3–70)	<0.05
Massive transfusion rate	13.5%	30%	
Infectious complications			
Nosocomial infections	56/104 (53.9%)	5/10 (50%)	
Sepsis	15/104 (14.0%)	5/10 (50%)	

Parameter	Discovery set (n = 10 patients)		*P*-value
	Systemic inflammation (n = 5)	Sepsis (n = 5)	
Demographics			
	mean ± SEM; median (min.-max.)	mean ± SEM; median (min.-max.)	
Age (yr)	34 ± 6.5; 28 (20–58)	48 ± 6.4; 47 (26–62)	0.21; ns
Sex (male/female)	4/1	4/1	
GCS	12.6 ± 1.9; 14 (5–15)	13 ± 1.6; 15 (7–15)	0.82; ns
Traumatic brain injury	2/5 (40%)	0/5 (0%)	
AIS max.	4.4 ± 0.4; 5 (5–3)	4.8 ± 0.2; 5 (4 -5)	
AIS Head/neck/cervical spine	2 ± 0.9; 2 (5–0)	1 ± 0.5; 1 (0–3)	
AIS Face	0.6 ± 0.4; 0 (2–0)	0.4 ± 0.2 0 (0–1)	
AIS Thorax/thoracic spine	2.6 ± 0.9; 3 (0–5)	4 ± 0.5; 4 (3–5)	
AIS Abdomen/lumbar spine	2.8 ± 0.9; 3 (0–5)	3.6 ± 0.7; 4 (2–5)	
AIS Upper/lower Extremitiy	2.2 ± 0.4; 2 (1–3)	2.8 ± 0.8; 3 (0–5)	
AIS Integument	1 ± 0.5; 1 (0–2)	0.4 ± 0.4; 0 (0–2)	
ISS	33.8 ± 2.0; 33 (27–38)	41.2 ± 3.7; 38 (34–50)	0.14; ns
SOFA score initial	4.8 ± 1.0; 5 (1–7)	6.2 ± 1.6; 7 (0–9)	0.20; ns
SOFA score max.	7.8 ± 1.8; 9 (1–12)	14 ± 0.6; 14 (12–16)	<0.05
Outcomes			
RISC (% survival)	92.4 ± 4.1; 96.7 (76.1–97.6)	83.9 ± 5.6; 83.4 (71.1–97.6)	0.38; ns
Survival	100%	100%	
Hospital length of stay (d)	27 ± 4.1; 25 (17–42)	53.8 ± 7.3; 49 (43–82)	<0.05
Intensive care unit length of stay (d)	17.4 ± 6.4; 12 (6–42)	37.4 ± 12.5; 26 (18–86)	0.10; ns
Allogeneic blood transfusion			
TASH score (points)	8.6 ± 2.2; 10 (2–15)	18 ± 4; 18 (14–22)	0.17; ns
TASH (%)	11 ± 4.6; 8 (5–29)	50 ± 27; 50 (23–77)	0.17; ns
Initial (d0) pRBC transfusion (units)	2.6 ± 0.8; 3 (0–5)	20.9 ± 9.4; 19 (0–54)	0.12; ns
Total pRBC transfusion (units)	6.8 ± 0.9; 6 (5–9)	35.6 ± 13.7; 24 (3–70)	0.14; ns
Massive transfusion rate	0%	60%	
Infectious complications			
Nosocomial infections	0/5 (0%)	5/5 (100%)	
Sepsis	0/5 (0%)	5/5 (100%)	

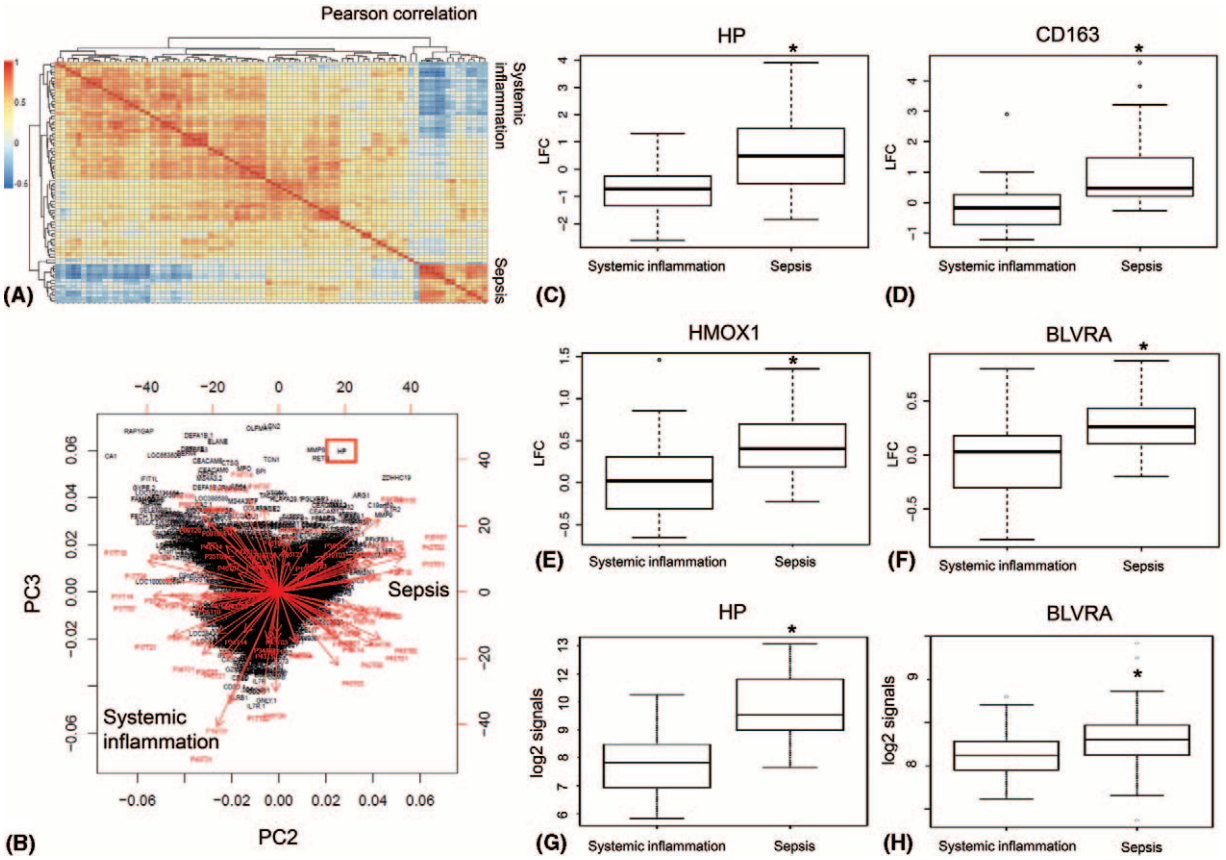

FIGURE 1. Whole-genome analysis of circulating leukocytes from trauma patients (n = 90 samples; 10 patients; all time points: day 0, 1, 2, 3, 5, 7, 10, 14, 21) who clinically either presented with systemic inflammation without infection (n = 5) or sepsis (n = 5). (A) Unsupervised cluster analysis (Pearson correlation; LFC values). (B) Principal component analysis characterizing differential gene expression profiles of trauma patients. Quantification of expression (logarithmic fold change, LFC; adjusted to day 0) of HP (C), CD163 (D), HMOX1 (E), BLVRA (F), and absolute expression values for HP (G) and BLVRA (H), comparing patients with systemic inflammation or sepsis. $^{*}P < 0.05$; NS = not significant.

Moreover, principle component analysis also revealed distinct expression patterns of patients with sepsis and patients with systemic inflammation without infection, and indicated an extraordinary role of haptoglobin (HP) in patients with sepsis among all genes analyzed (Fig. 1B). In line with the findings of the discovery set, re-investigation of data sets from external, independent cohorts also identified HP among top 10 upregulated genes in patients who developed sepsis in comparison to healthy controls or patients with SIRS without infection by one-way ANOVA ($P < 10^{-12}$).[26]

For further analysis, expression of the key components of the heme degradation pathway HP (Fig. 1C), cluster of differentiation 163 (CD163; Fig. 1D), heme oxygenase-1 (HMOX1; Fig. 1E), and biliverdin reductase A (BLVRA; Fig. 1F) was quantified. All genes of the heme degradation pathway showed a similar pattern with significant upregulation in patients with sepsis as compared to patients with systemic inflammation without infection, as for normalization to time point d0 (Fig. 1C–F) or when comparing absolute expression values (Fig. 1G and H). Furthermore, these expression

TABLE 2. Explorative Gene-Set Analysis

Knowledge Base	Category Name	# Hits	*P*-value (Fisher Exact Test)	FDR-adjusted *P* (Benjamini-Hochberg)
Upregulated genes: average $\log_2$FC (sepsis versus systemic inflammation) >0.5				
Gene Ontology CC	Hemoglobin complex	10	1.60E-11	6.00E-09
Swiss Prot	Oxygen transport	8	2.10E-08	3.50E-06
KEGG PATHWAY	Pathogenic E. coli infection	8	2.90E-06	4.00E-04
Gene Ontology BP	Hemoglobin metabolic process	5	3.00E-04	5.80E-02
Downregulated genes: average $\log_2$FC (sepsis versus systemic inflammation) < −0.5				
Gene Ontology BP	Translational elongation	26	5.00E-14	6.90E-11
Swiss Prot	Protein biosynthesis	31	1.30E-12	3.00E-10
KEGG PATHWAY	Ribosome	25	2.20E-12	3.30E-10
Gene Ontology BP	Defense response	65	2.40E-13	2.20E-10

patterns were confirmed by external validation of two independent cohorts (datasets: GPSSSI unique and GSE9960) of a recent multicohort analysis, comparing patients with sterile inflammation (SIRS/trauma) to time-matched patients with infections by whole-genome screening (Supplemental Fig. 3, http://links.lww.com/SLA/A929).[26–28]

Effect of Allogeneic Blood Transfusion on Clinical Outcome Parameters

Based on the results from whole-genome screening of the discovery set, samples from all patients with complete clinico-transcriptomic coverage (n = 71; 517 samples) were analyzed for gene expression patterns of HP, CD163, HMOX1, BLVRA, and BLVRB by quantitative RT-PCR normalized to the housekeeping gene ACTB (ΔCt). In a first step, unsupervised clustering was applied to clinical parameters and expression of the key components of the heme degradation pathway. As shown in Fig. 2A, HP clustered with BLVRA. As to be expected, the total blood transfusion rate (pRBC-total) correlated with the Trauma Associated Severe Hemorrhage (TASH) score and the hematocrit (Fig. 2A). Among the scoring systems, the RISC score negatively correlated with the ISS, the New Injury Severity Score (NISS), and the SOFA score (Fig. 2A). Interestingly, HP, CD163, HMOX1, BLVRA, and BLVRB collectively clustered with the ICU length of stay (ICU-LOS). Since the heme degradation pathway is activated by free heme, the initial and total amount of packed red blood cells (pRBCs) transfusion was assessed for various outcome parameters. Trauma patients who developed nosocomial infections received more units of pRBCs (initial = at the day of trauma; as well as total) than patients without infection (Fig. 2B). These differences in the amount of allogeneic blood transfusion were even more striking in sepsis patients as compared to trauma patients who did not develop secondary sepsis (Fig. 2C). In contrast, there was no difference in the initial and total volume of allogeneic blood transfusion in patients who did not survive in comparison to survivors (Fig. 2D).

In the present patient cohort, the TASH score as a measure for the risk of massive blood transfusion (≥10 pRBC within 24 hours) matched with the actual mass transfusion rate (Table 1). In accordance with Fig. 2B and C, trauma patients with nosocomial infections or sepsis had significantly higher TASH scores than patients without infectious or septic complications (Fig. 2E and F).

Furthermore, the volume of allogeneic blood transfusion correlated with the ICU-LOS, as displayed in Fig. 2G for the total amount as well as for the initial amount of pRBC administered

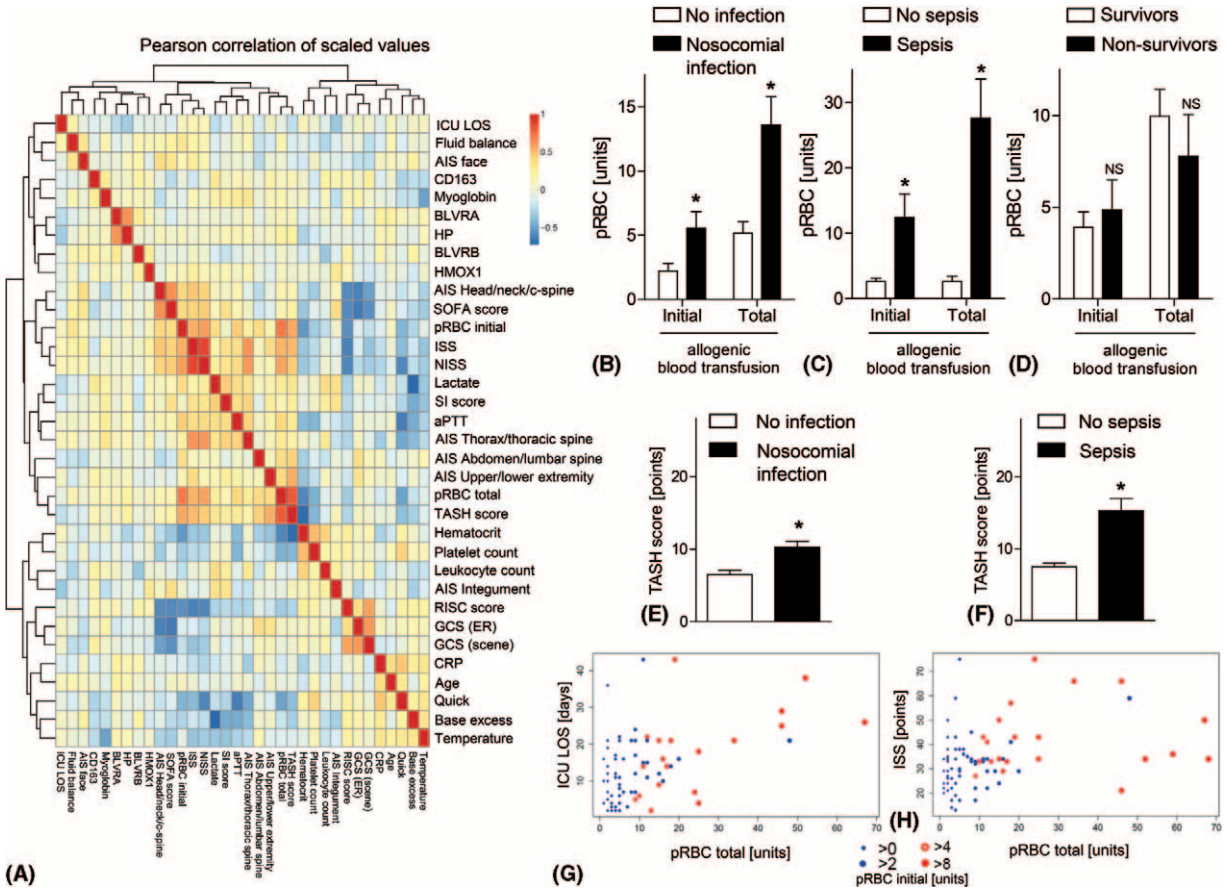

FIGURE 2. Correlation of clinical parameters of trauma patients (n = 52) and expression of heme degradation pathway expression (ΔCt of HP, CD163, HMOX1, BLVRA, and BLVRB) (A). Association of transfusion of packed red blood cells (pRBCs; total vs. initial amount = at day 0) with nosocomial infections (B), sepsis (C), and survival (D). Comparison of TASH scores of patients with nosocomial infections (E) or sepsis (F) to trauma patients without infectious complications. N = 104; $^{*}P < 0.05$; NS = not significant. Correlation of the total amount of pRBC transfusion of with the ICU-LOS (G) and ISS (H). Initial amount of pRBC transfusion is color coded (blue represents ≤4 units; red represents >4 units).

(Spearman correlation coefficient ρ [total pRBC] = 0.55; ρ [initial pRBC] = 0.39). However, there was also an association between the amount of pRBC transfusion and the ISS (Fig. 2H; ρ [total pRBC] = 0.43; ρ [initial pRBC] = 0.44). Similarly, trauma patients who developed nosocomial infections or sepsis had higher ISS than patients without infections or sepsis, respectively (Supplemental Fig. 4A and B, http://links.lww.com/SLA/A929). With respect to massive blood transfusion, higher TASH scores were associated with higher ISS (Supplemental Fig. 4C, http://links.lww.com/SLA/A929). However, there was no significant difference in the ISS in patients who did not receive allogeneic blood transfusion at all in comparison to patients who received 1 unit of pRBC or more (Supplemental Fig. 4D, http://links.lww.com/SLA/A929). Notably, none of the patients without allogeneic blood transfusion developed secondary sepsis.

Analysis of the Expression Patterns of the Heme Degradation Pathway

Cluster analysis of the expression of HP, CD163, HMOX1, and BLVRA as determined by microarray (discovery set; Fig. 3A) or quantitative real-time PCR (validation set; Fig. 3B) in which samples were arranged according to the clinical presentation of sepsis or systemic inflammation revealed a distinct expression pattern of HP in sepsis.

Correlation of Heme Degradation Pathway Expression with Changes in the Severity of Systemic Inflammation and the Development of Sepsis

For further correlational analysis of gene expression data with the clinical course, the SI score for assessment of the severity of systemic inflammation has been applied. As shown in Fig. 4, expression patterns of components of the heme degradation pathway followed the clinical course (SI score). Whereas shifts toward increased severity of systemic inflammation was associated with an upregulation of the heme degradation pathway components HP (Fig. 4A; ANOVA $P < 10^{-10}$, ρ [SI score, HP] = 0.44) and HMOX1 (Fig. 4C), the expression of BLVRA (Fig. 4D) was downregulated with increasing SI score. These linear trends were most profound for the expression of HP (Fig. 4A). With respect to the hierarchy of the heme degradation cascade, the ratio of BLVRA and HMOX1 decreased with increasing SI score, suggesting that BLVRA becomes the rate-limiting step in higher grades of systemic inflammation (Fig. 4E). Moreover, the strongest upregulation of HP expression was found in patients who were diagnosed with sepsis (Fig. 4F), further corroborating the association between expression of the heme degradation pathway and the development of sepsis. In these patients, aggravation of systemic inflammation (positive ΔSI score) was accompanied by an upregulation of HP expression, whereas

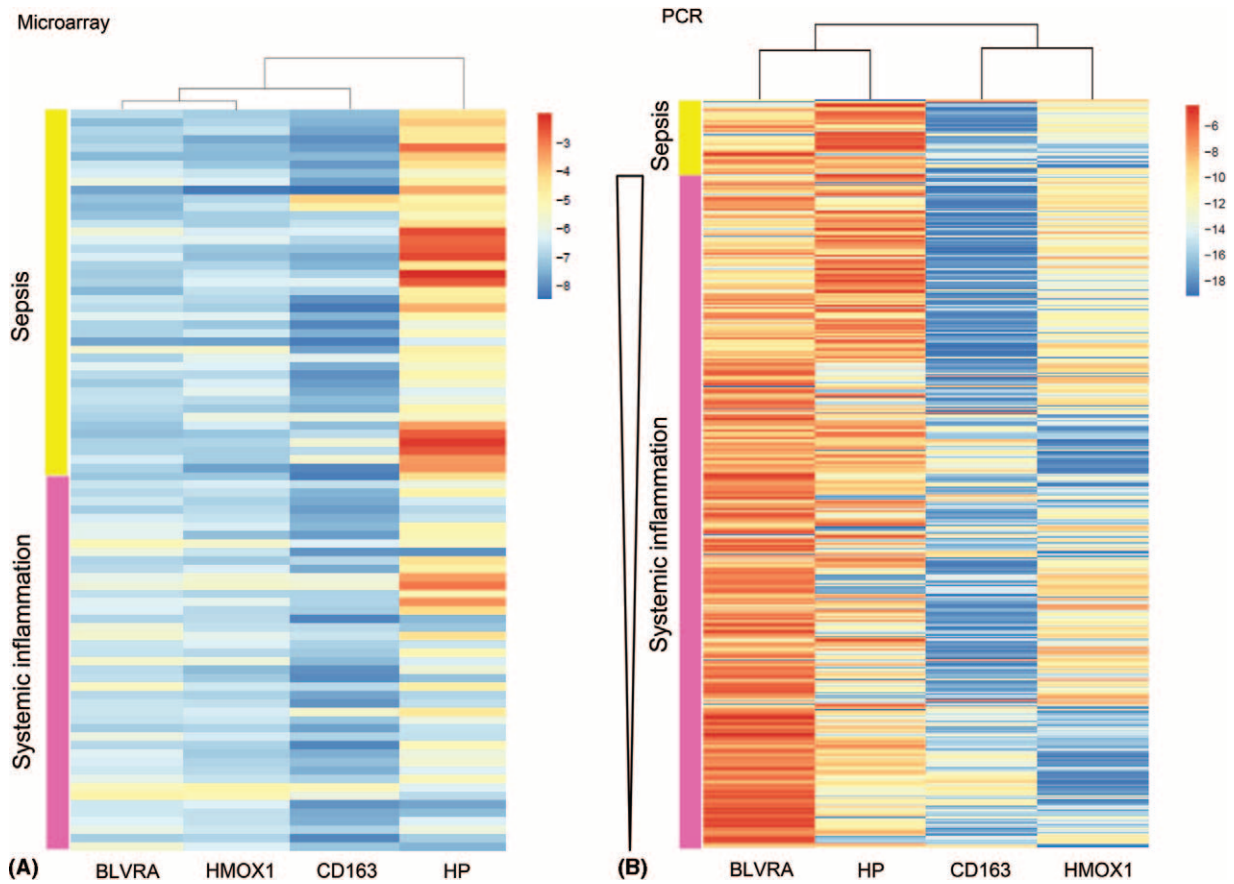

FIGURE 3. Correlation (cluster analysis) of the expression of HP, CD163, HMOX1, and BLVRA of the discovery set (A; microarray; n = 90 samples; 10 patients; all time points) and the validation set (B; quantitative RT-PCR; n = 517 samples; 71 patients; all time points) arranged by clinical presentation: sepsis vs. systemic inflammation.

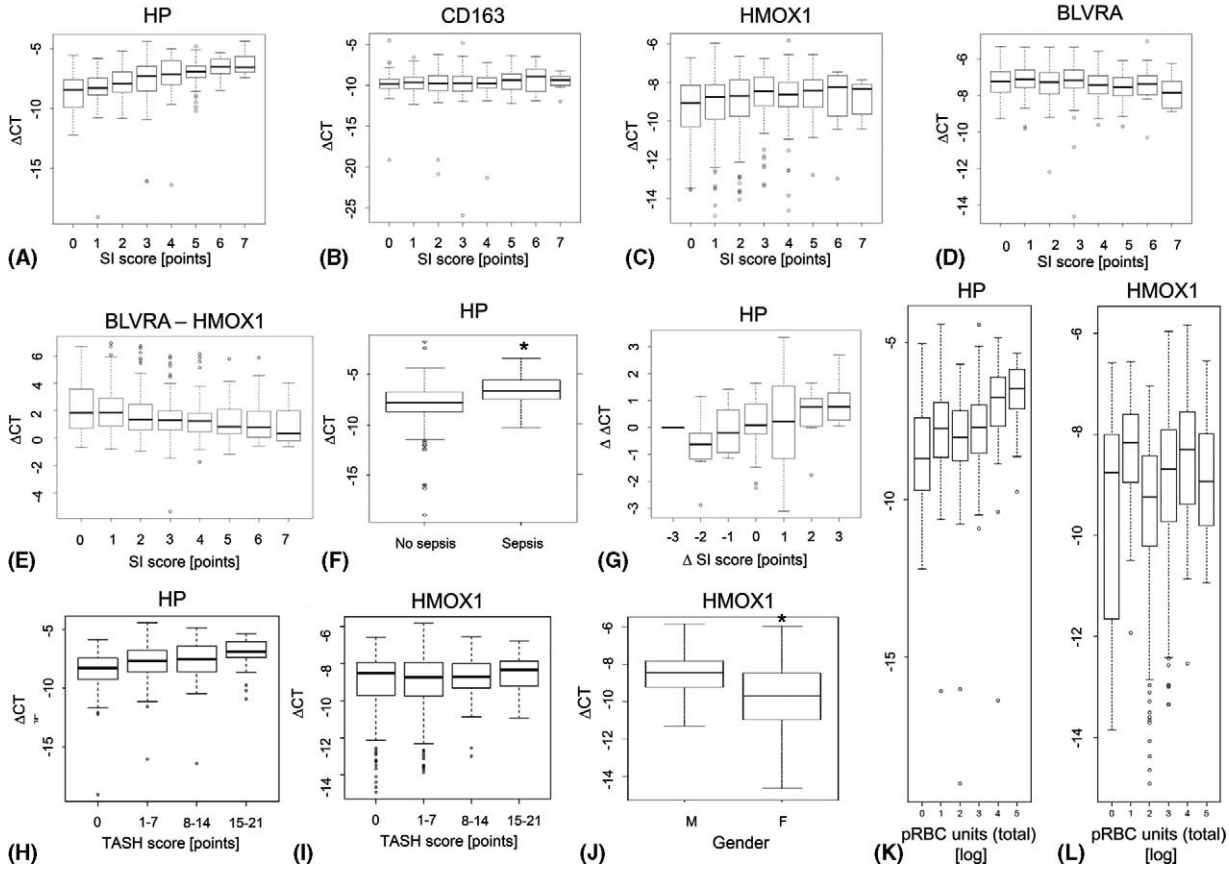

FIGURE 4. Expression (ΔCt) of HP (A), CD163 (B), HMOX1 (C), and BLVRA (D) in correlation with the severity of systemic inflammation (SI score). Significance, using $P < 0.05$ from ANOVA and strong linear and nonlinear correlation (rho > 0.4) was obtained for HP. (E) Hierarchical relationship between HMOX1 and BLVRA in correlation with the SI score. (F) Expression of HP (ΔCt) in trauma patients with sepsis as compared to patients without sepsis. (G) Correlation of changes in HP expression (ΔΔCt = difference between consecutive time points) with corresponding changes in severity of systemic inflammation (ΔSI score). Association of the TASH score with the expression (ΔCt) of HP (H) and HMOX1 (I). (J) Influence of gender on HMOX expression (ΔCt). Relationship between the amount of allogeneic blood transfusions (log number of total pRBCs) and the expression (ΔCt) of HP (K) and HMOX1 (L). N $\geq$ 51 patients. $^{*}P < 0.05$.

attenuation (negative ΔSI score) went along with downregulation of HP (Fig. 4G).

Interestingly, an increasing risk for massive transfusion (TASH score) was reflected by an upregulation of HP (Fig. 4H), whereas there were no changes in the expression of HMOX1 (Fig. 4I). As a possible explanation for the latter observation, there was a strong gender effect on the expression of HMOX1 (Fig. 4J). Similar to the TASH score, HP expression but not HMOX1 correlated with the amount of allogeneic blood transfusions (number of total pRBCs; Fig. 4K and L). In accordance, systematic, multivariate factorial analysis (MANOVA; Supplemental Table 4, http://links.lww.com/SLA/A929) showed that HP positively correlated with the rate of allogeneic blood transfusions, SI score, and sepsis. Similarly, HMOX1 expression was influenced by the total amount of pRBCs and associated with sepsis. Interestingly, these analyses showed significant time effects for BLVRA and CD163. A complete list of *P* values is provided in Supplemental Table 4, http://links.lww.com/SLA/A929.

In further correlational analyses of heme degradation pathway expression and various mediators of inflammation (Supplemental Fig. 5, http://links.lww.com/SLA/A929), HP expression showed a stronger positive correlation with the anti-inflammatory cytokine IL-10 than with the pro-inflammatory mediators IL-8 or TNF-α, suggesting that upregulation of the heme degradation pathway may be related to a rather anti-inflammatory phenotype.

Temporal Relationship between Gene Expression and the Development of Sepsis

In order to depict temporal changes of heme degradation pathway gene expression relative to clinical events, trajectories of septic patients were plotted in relation to the expression of the total cohort (discovery set: Fig. 5; Supplemental Fig. 6, http://links.lww.com/SLA/A929; validation set: Supplemental Figs. 7 and 8, http://links.lww.com/SLA/A929). Although the courses were found to be highly individual for each patient, collectively, administration of allogeneic blood was followed by an upregulation of heme degradation pathway expression, and continuous increases or secondary peaks preceded the clinical diagnosis of sepsis.

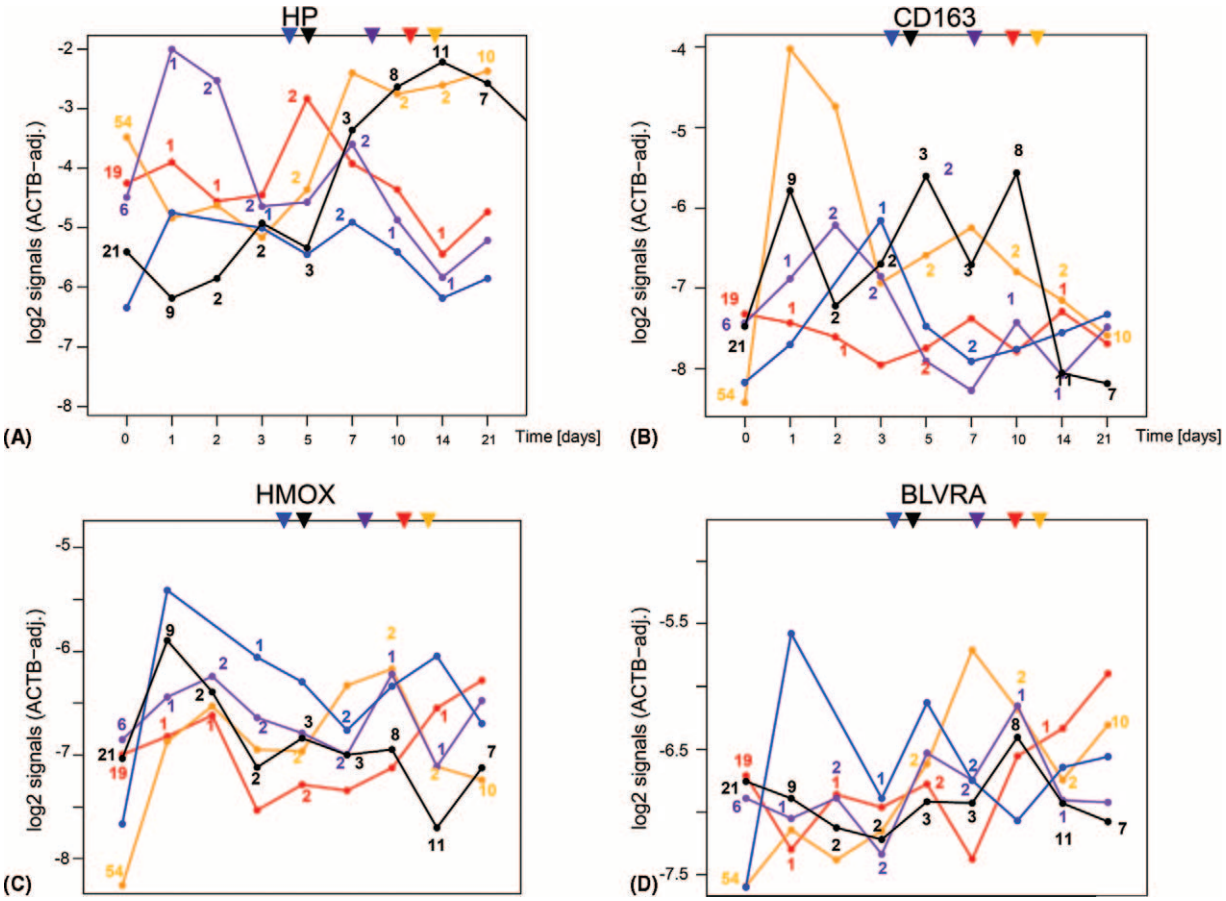

FIGURE 5. Temporal relationship of the expression of HP (A) CD163 (B), HMOX1 (C), and BLVRA (D) with the clinical course. The individual course of each patient with sepsis (n = 5) of the discovery set (n = 90 samples; 10 patients) are plotted as trajectories in different colors. The time points of diagnosis of sepsis are indicated by the arrows on the top of each figure. The amount of allogeneic blood transfusions (number of pRBCs) is indicated at specific time points for each patient.

DISCUSSION

The present study demonstrates for the first time that the heme degradation pathway is differentially regulated after multiple trauma and is associated with septic complications. The expression of HP in particular indicates the severity of systemic inflammation and represents a promising marker for secondary sepsis in trauma patients.

Our data suggest that the heme degradation pathway is triggered by free heme released from allogeneic blood transfusions after hemorrhagic shock, which was also associated with a significantly higher incidence of nosocomial infections and sepsis and a prolonged stay on the ICU. This is in line with several previous studies reporting that the transfusion of allogeneic blood represents an independent risk factor in critically ill patients for the development of infectious complications, MOF, and even mortality.[1,12,29–33] With respect to the latter, a recent study demonstrated that only patients with a high risk of death due to traumatic hemorrhagic shock benefit from pRBC transfusions, whereas mortality is increased in trauma patients with a low risk of death.[33] In our cohort, the expression patterns of the heme degradation pathway did not correlate with mortality, suggesting that the transcriptomic signatures are specific for the development of secondary sepsis in trauma patients but do not relate to an adverse outcome in general. Both the TASH score and amount of pRBC transfusion correlated with the development of sepsis and the HP expression pattern, corroborating the relationship between blood transfusion, regulation of the heme degradation pathway, and the development of sepsis. However, due to the clinical–translational nature of the study and multiple dependent variables, causal pathophysiologic relationships are difficult to interpret, and it remains unclear whether the changes of gene expression are secondary to blood transfusions or to the condition of hemorrhagic shock itself.

It is well established that pRBCs can undergo nonimmunologic hemolysis due to mechanical injury of RBCs and/or RBC storage lesions, resulting in an increased cellular fragility. Depending on the age of the pRBC product, up to 25% of the RBCs undergo nonimmunologic hemolysis within 24 hours after transfusion, resulting in a considerable amount of heme.[34] In experimental sepsis, free heme has been described to play a central role by compromising the host tolerance to infection.[35] Based on findings of the present study and published mechanisms of the heme degradation pathway, we conceptualize the pathophysiology in trauma as follows: free heme released from hemoglobin after allogeneic blood transfusions represents a robust proinflammatory stimulus and functions as a danger-associated molecular pattern, but it also exerts cytotoxic effects.[36–38] In order to neutralize free heme, the heme-degradation pathway is activated, which may shift the balance of the immune response toward an anti-inflammatory phenotype.[39] HP as the

immediate interaction partner of free heme and HMOX1 as the rate-limiting step of the enzymatic heme degradation cascade represent key components of the heme degradation pathway and are known as key regulators of the immune response.[35,40–43] Prolonged dysregulation of the heme-degradation pathway may contribute to an increased susceptibility of secondary infections and the development of sepsis. On the contrary, it is conceivable that in the case of an overwhelming release of free heme, mechanisms for neutralization and degradation of heme may become saturated, resulting in remote tissue damage by circulating free heme and a reduced tolerance to infection. Experimental and clinical studies suggest that HMOX1 is the rate-limiting step of heme degradation, and that its genotypes and underlying regulatory mechanisms are associated with the outcome in sepsis.[44–47]

The particular design of the present study with purposeful selection of patients for the discovery set (secondary sepsis vs. systemic inflammation without infection) and consecutive validation of candidates in the total cohort by an independent method has been conceptualized to overcome possible masking effects by ''non-selective'' whole-genome screening of all patients. In contrast to our results, gene expression patterns in a similar trauma patient cohort in a previous study by the Glue grant consortium were found to be more common than different in patients with ''complicated'' or ''uncomplicated recovery.''[13] As a matter of speculation, this rather unspecific distribution of patients into subgroups might have contributed to masking of differential gene expression. This assumption is supported by the fact that HP was also found among the genes whose expression increased the most in the ''genomic storm'' study.[13] In fact, similar correlations of heme degradation pathway expression with clinical variables as in our study were found reanalyzing the publicly available Glue grant data set (eg, correlation of HP expression with volume of allogeneic blood transfusions, incidence of nosocomial infections and SSI, or ICU-LOS; data not shown) and additional data sets of two independent cohorts that are part of a recent multicohort analysis, comparing patients with sterile inflammation to sepsis.[13,26–28]

In our cohort as well as in external data sets, HP expression has been consistently identified as a promising marker for sepsis in trauma patients, which may even be superior to and more specific than serum bilirubin, as HP expression precedes the generation of bilirubin, and high bilirubin levels can not only be caused by increased heme degradation but also by impaired hepatic secretion.[48]

Our data indicate that an integrated clinico-transcriptomic approach facilitates to distinguish between systemic inflammation without infection and sepsis after trauma, in accordance with previous studies in trauma and nontrauma patients with sepsis.[26,49–51] With respect to the impact on the treatment strategy of trauma patients, our findings imply that patients who received blood transfusions should be closely monitored regarding the development of septic complications using specific markers. In future, routine evaluation of trauma patients may ideally comprise integrated assessment of clinical, laboratory, proteomic, and transcriptomic parameters, on the basis of which treatment algorithms, including timing of secondary surgical interventions as well as operative techniques to be applied, could be adapted to the individual risk profile. The expression patterns of the heme degradation pathway in leukocytes, with HP in particular, may help assess the host immune status and response and stratify the individual risk of trauma patients for infectious complications and sepsis.

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